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Smith–Lemli–Opitz Syndrome Produced in Rats With AY 9944 Treated by Intravenous Injection of Lipoprotein Cholesterol

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A limitation to treating Smith–Lemli–Opitz infants by giving dietary cholesterol is their impaired ability to absorb cholesterol due to a deficiency of bile acids. Since intravenously administered lipoprotein cholesterol should not require bile acids for uptake into tissues, we tested the effects of this form of cholesterol on tissue cholesterol and 7-dehydrocholesterol levels in an animal model of SLO, created by feeding rats 0.02% AY 9944. Intravenous administration of 15 mg of bovine cholesterol supertrate twice daily increased serum cholesterol levels from 11 to over 250 mg/dl. This treatment increased liver cholesterol levels from 309 to over 900 µg/g and lowered hepatic 7-dehydrocholesterol levels from 1546 to 909 µg/g. A combination of iv cholesterol and 2% dietary cholesterol was most effective as it raised hepatic cholesterol levels to 1950 µg/g, which is 50% above normal. 7-Dehydrocholesterol levels were decreased to 760 µg/g. Similar responses were seen for heart, lung, kidney, and testes. Brain sterol levels were not significantly affected. AY 9944 caused a modest increase in hepatic HMG-CoA reductase activity. Administration of dietary cholesterol together with iv cholesterol lowered hepatic HMG-CoA reductase activity to barely detectable levels. The data indicate that the combination of iv and dietary cholesterol was most effective in raising cholesterol levels, lowering 7-dehydrocholesterol levels, and inhibiting de novo cholesterol biosynthesis. Am. J. Med. Genet. 68:322–327, 1997.

KEY WORDS: Smith–Lemli–Opitz syndrome; AY 9944; intravenous lipoprotein cholesterol

INTRODUCTION

The Smith–Lemli–Opitz syndrome (SLOS) [Smith et al., 1964] is a recessively inherited birth defect with an estimated occurrence of 1 in 40,000 to 1 in 20,000 [Lowry and Young, 1980], comprising microcephaly, ptosis, wide nasal bridge with anteverted nares, apparently low-set posteriorly angulated ears, and micrognathia, syndactyly of toes 2 and 3, multiple organ congenital anomalies, and severe mental retardation [Irons et al., 1995]. The biochemical defect responsible for SLO was identified by Tint et al. [1994]. These investigators showed that serum and tissues from SLO subjects had very low cholesterol levels with more than a 2000-fold elevation in the level of the cholesterol precursor 7-dehydrocholesterol. Subsequently, it was demonstrated that SLO patients have reduced levels of 7-dehydrocholesterol-Δ^7-reductase, the enzyme which converts 7-dehydrocholesterol to cholesterol [Salen et al., 1995]. Cholesterol is an essential component of cell membranes and precursor of bile acids and steroid hormones. Therefore, it has been suggested that the organ dysfunction of SLO subjects arises from the lack of available cholesterol and the substitution of 7-dehydrocholesterol for cholesterol.

Different treatment strategies have been implemented in order to supply tissues with cholesterol. These strategies involve administration of dietary cholesterol, or dietary cholesterol plus the bile acids, ursodeoxycholic acid and chenodeoxycholic acid [Irons et al., 1995]. It has been demonstrated that SLO patients have very low levels of normal bile acids and have several abnormal urinary bile acids [Natowicz

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et al., 1994]. The cholesterol administered would require endogenously synthesized bile acids for absorption and would be taken up predominantly by the liver through the chylomicron remnant receptor pathway. This would supply the liver with cholesterol and serve to down-regulate HMG-CoA reductase, the enzyme which catalyzes the rate-limiting step of cholesterol biosynthesis, thereby lowering hepatic 7-dehydrocholesterol levels. In the case of one severely affected individual that we have examined, profound hyperbilirubinemia was found, indicating a lack of bile acids due to the deficiency of 7-dehydrocholesterol-Δ^7-reductase [Ness et al., 1997]. Treatment with dietary cholesterol of an individual with low bile acid levels may be inefficient in supplying tissues with cholesterol. Therefore, we have investigated the effectiveness of intravenously administered lipoprotein cholesterol in raising serum and tissue cholesterol levels and lowering 7-dehydrocholesterol levels using rats treated with AY 9944, an inhibitor of 7-dehydrocholesterol-Δ^7-reductase, as a model of SLOS. Previously, we used BM 15.766 [Xu et al., 1995]. Both compounds are effective inhibitors of the conversion of 7-dehydrocholesterol to cholesterol.

METHODS

Male Spraque–Dawley rats weighing 100–125 g were purchased from Harlan Industries of Madison, WI. The animals were housed in a windowless reverse light cycle room. Lights were on from 3 P.M. to 5 A.M. and off from 5 A.M. to 3 P.M. Animals were allowed free access to Purina Laboratory Chow and water. To establish the SLOS model, animals were fed 0.02% AY 9944 for 4 days. AY 9944 was a generous gift from Wyeth-Ayerst. Animals were then divided into four different groups. Each group continued to receive AY 9944 throughout the experiment. The first group served as a control and received 0.02% AY 9944 in chow only. For the second group, 2% dietary cholesterol was added at 8 A.M. on day 5. The third group was treated with 15 mg cholesterol supertrate per animal by intravenous injection via the jugular vein under ether anesthesia at 8 A.M. and 5 P.M. on days 5, 6, and 7, and at 8 A.M. on day 8. Cholesterol supertrate contains 3015 mg/dl of bovine lipoprotein bound cholesterol and was purchased from Pentex/Bayer Corporation Diagnostics Division, Kankakee, IL. The fourth group was treated the same as group three with the addition of 2% cholesterol to the chow at 8 A.M. on day 5. All animals were killed at 9 A.M. on day 8. Each group contained at least 3 animals.

Levels of cholesterol and 7-dehydrocholesterol were determined by reverse-phase HPLC. Approximately 100 mg of tissue was removed and saponified in 1 ml of 60% KOH/MeOH at 100°C for 1 hr. For the purpose of recovery 90,000 cpm of [^{3}H]cholesterol was added. Extraction was done twice with 4 ml of petroleum ether each time. Extracts were combined and dried under argon. Dried samples were dissolved in 1 ml of MeOH. Samples, 100 µl, were resolved on a Spherei-5, RP-18, 5-µm reverse-phase column. Elution was done with 100% methanol. Identification of sterols was done by comparison to standards. Quantification was performed by monitoring the absorbance at 210 nm and comparing with the absorbance of the known compounds. Values are expressed as µg/g.

Low-density lipoprotein receptor protein was detected by immunoblotting analysis. Liver microsomal protein was separated on 7.5% SDS polyacrylamide gels in the presence of 2-mercaptoethanol. Protein was then transferred to Hybond nitrocellulose membranes. Membranes were blocked using 5% nonfat dry milk. Membranes were then incubated at room temperature with a 1:1000 dilution of antirat low-density lipoprotein receptor serum. Immunoreactive protein was detected using the Vistra ECF substrate system with antirabbit alkaline phosphatase-linked secondary antibody purchased from Amersham Life Science and the Storm 860 imaging system purchased from Molecular Dynamics.

HMG-CoA reductase activity was measured in hepatic microsomes using [^{14}C]HMG-CoA and an NADPH regenerating system as described previously [Ness et al., 1987]. The concentration of protein was determined by a biuret assay. Each reaction contained 100 µg of protein in a volume of 100 µl. Reactions were incubated for 10 min and the product, [^{14}C]mevalonate, was converted to mevalonolactone and isolated by thin-layer chromatography. Enzymatic activity is expressed in nmol/min/mg of protein and then converted to relative activity for comparative purposes.

RESULTS

Because endogenously synthesized bile acids are necessary for absorption of dietary cholesterol and could be diminished in an SLO patient, we determined serum and tissue sterol levels in AY 9944 treated rats after intravenous administration of lipoprotein cholesterol. Results are shown in Figure 1. Serum and tissue sterol levels of rats given only intravenous lipoprotein cholesterol or only dietary cholesterol or both are compared. Levels of 7-dehydrocholesterol (7-DHC) and cholesterol (CH) levels are given for serum (mg/dl) and tissues (µg/g). Experimental conditions are given above panels A and B. Administration of iv cholesterol resulted in more than a 20-fold increase in serum cholesterol levels (Fig. 1A). Dietary cholesterol caused only a 2-fold increase. When both were given no additive affect was seen. Interestingly, serum 7-dehydrocholesterol levels were actually increased by these treatments rather than reduced. Perhaps this reflects transport of 7-dehydrocholesterol out of tissues where cholesterol synthesis is being inhibited by AY 9944. In liver, both dietary and iv cholesterol administration increased cholesterol levels over 2-fold. However, the combined treatment was most effective in increasing hepatic cholesterol. It also reduced hepatic 7-dehydrocholesterol by 50% (Fig. 1B). This same pattern was also observed in heart tissue (Fig. 1C). In lung, kidney, and testes, iv cholesterol administration was more effective that dietary cholesterol (Fig. 1E, F, and G). In brain the treatments had very little effect (Fig. 1D). This may reflect the slow turnover of cholesterol in rats of this age. Maximal rates of cholesterol synthesis occur in rat brain at about age 4 days [Ness, 1994].

To better analyze the effectiveness of administration of cholesterol, the ratios of cholesterol to 7-dehydrocho-

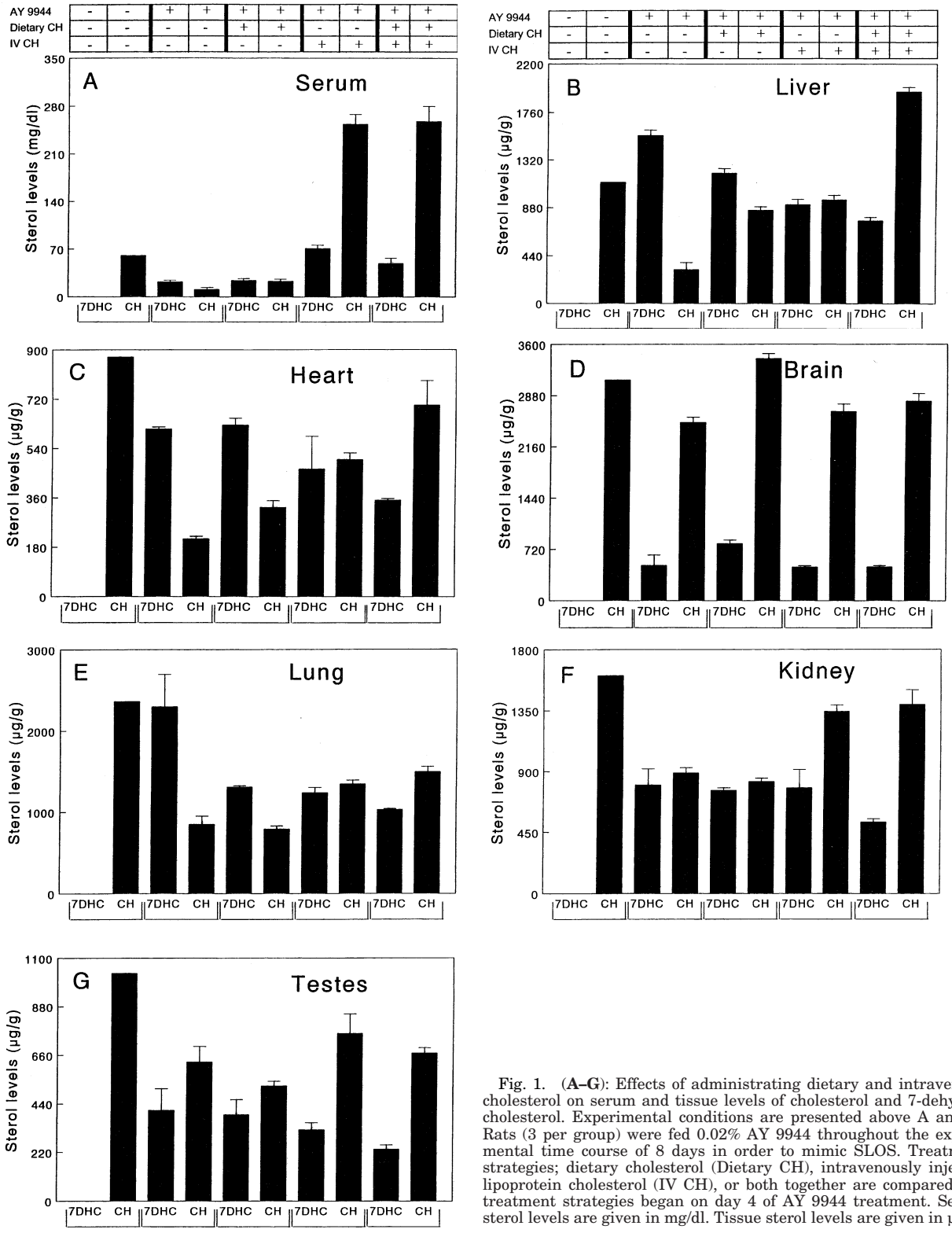

Fig. 1. (**A–G**): Effects of administrating dietary and intravenous cholesterol on serum and tissue levels of cholesterol and 7-dehydrocholesterol. Experimental conditions are presented above A and B. Rats (3 per group) were fed 0.02% AY 9944 throughout the experimental time course of 8 days in order to mimic SLOS. Treatment strategies; dietary cholesterol (Dietary CH), intravenously injected lipoprotein cholesterol (IV CH), or both together are compared. All treatment strategies began on day 4 of AY 9944 treatment. Serum sterol levels are given in mg/dl. Tissue sterol levels are given in µg/g.

TABLE I. Ratio of Cholesterol to 7-Dehydrocholesterol in Serum and Tissues of AY-9944 Rats Treated With Either Dietary Cholesterol, iv Cholesterol, or Both*

Tissue	AY	AY + diet	AY + iv	AY + diet + iv
Serum	0.50	0.96	3.58	5.27
Liver	0.27	0.71	1.10	2.30
Heart	0.34	0.52	1.07	1.98
Brain	5.01	4.25	5.69	5.94
Lung	0.37	0.60	1.08	1.45
Kidney	1.11	1.08	1.70	2.66
Testes	1.53	1.33	2.53	2.85

* No ratios are presented for normal rats since 7-dehydrocholesterol levels are essentially unmeasureable.

lesterol were determined (Table I). A significant increase in the ratio is indicative of effective treatment. The combination of dietary and intravenous cholesterol increased the ratio of cholesterol to 7-dehydrocholesterol about 10-fold in serum and liver (Table I). Increases of 6- and 4-fold were seen in heart and lung, respectively. In kidney and testes the increase was 2-fold. In brain the ratio was essentially unaffected.

In view of the depletion of serum and tissue cholesterol levels caused by administration of AY 9944, we wished to determine whether there were compensatory increases in hepatic cholesterol biosynthesis or uptake of LDL. As shown in Figure 2a modest increase in the activity of hepatic HMG-CoA reductase, the enzyme which catalyzes the rate-limiting step in cholesterol biosynthesis, was observed in AY 9944-treated rats. In contrast, administration of HMG-CoA reductase inhibitors increase the levels of this protein 300-fold [Ness et al., 1994, 1996]. Administration of cholesterol in the diet or iv significantly lowered hepatic HMG-CoA reductase activity (Fig. 2). Administration of cholesterol in both forms was most effective at lowering HMG-CoA reductase activity (Fig. 2). Levels of immunoreactive HMG-CoA reductase protein changed in parallel with enzyme activity (data not shown). The effects of AY 9944 and cholesterol treatments on levels of

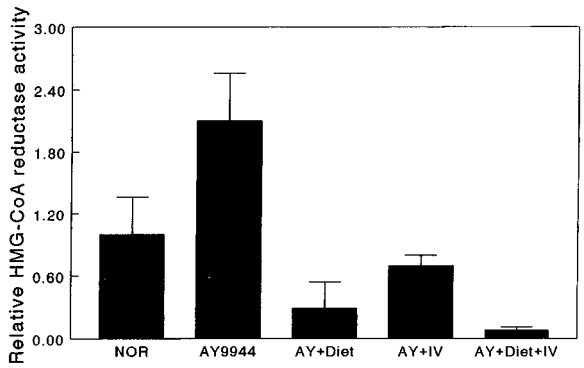

Fig. 2. Effects of dietary and intravenously administered cholesterol on hepatic HMG-CoA reductase activity in AY 9944-treated rats. Experimental conditions include Normal (NOR), 0.02% AY 9944 (AY 9944), AY 9944 plus 2% dietary cholesterol (AY + Diet), AY 9944 plus two daily intravenous injections of lipoprotein cholesterol (AY + IV), and AY 9944 plus both treatment methods (AY + Diet + IV). Administration of cholesterol began on day 4 of AY 9944 treatment. Activity is expressed as nmol/min/mg protein.

hepatic LDL receptor protein were also determined. As can be seen in Figure 3, no significant changes were observed. This is in contrast with the large increases seen in LDL receptor protein in brain and liver tissue from a severely affected SLO subject who presented with jaundice [Ness et al., 1997]. Perhaps the administration of AY 9944 to these adolescent rats does not provide as severe a depletion of cholesterol as seen in the subject. The ratio of cholesterol to 7-dehydrocholesterol in the brain of subject K.S. was 0.23 while in AY 9944 treated rats the ratio was 5.01 (Table I).

DISCUSSION

Using an animal model of SLO, we have evaluated the effectiveness of giving lipoprotein cholesterol intravenously alone and in combination with dietary cholesterol on restoring cholesterol levels and reducing levels of the precursor 7-dehydrocholesterol. The potential advantage of this form of treatment is that it is not dependent on endogenous bile acids. It was found that the combination of dietary and iv cholesterol was the most effective form of treatment. This treatment was beneficial for all tissues except brain in these adolescent rats.

It was surprising that treatment of these rats with AY 9944 did not cause a much larger increase in hepatic HMG-CoA reductase activity such as was observed with Lovastatin and other inhibitors of HMG-CoA reductase [Ness et al., 1994, 1996]. Previously we showed that BM 15.766, another inhibitor of 7-dehydrocholesterol-Δ^7-reductase, also causes modest increases in HMG-CoA reductase gene expression [Xu et al., 1995]. There are several possible explanations. Although liver cholesterol levels were reduced to a third, total sterols were actually higher in AY 9944-treated animals. Perhaps 7-dehydrocholesterol or a product derived from it exerts some degree of feedback regulation on HMG-CoA reductase. Another possibility is that feedback regulation is predominantly mediated by a metabolite derived from an intermediate in the pathway. Thus, AY 9944 would not block the production of this metabolite. In this regard, it has been shown that an oxygenated lanosterol compound, 3β-hydroxylanost-8-en-32 al, is a potent regulator of HMG-CoA reductase gene expression at the translational level [Leonard et al., 1994; Trzaskos, 1995]. Dietary cholesterol also appears to regulate HMG-CoA reductase at the level of translation [Ness et al., 1991, 1994].

In contrast with the marked elevation in hepatic LDL receptor protein seen in the severely affected SLO patient described by Ness [1997], AY 9944-treated adolescent rats showed no increase in receptor immunoreactive protein levels (Fig. 3). This may reflect the fact that total hepatic sterols are not depleted in such rats. It would appear that these AY 9944-treated rats are not severely affected. The animals had normal weight gains indicating no feeding difficulties. To generate a comparable severely affected animal model, administration of inhibitors such as AY 9944 or BM 15.766 [Xu et al., 1995] may have to begin in utero. However, this treatment frequently leads to stillborn animals.

From the data presented, it would appear that administration of iv lipoprotein cholesterol alone or to-

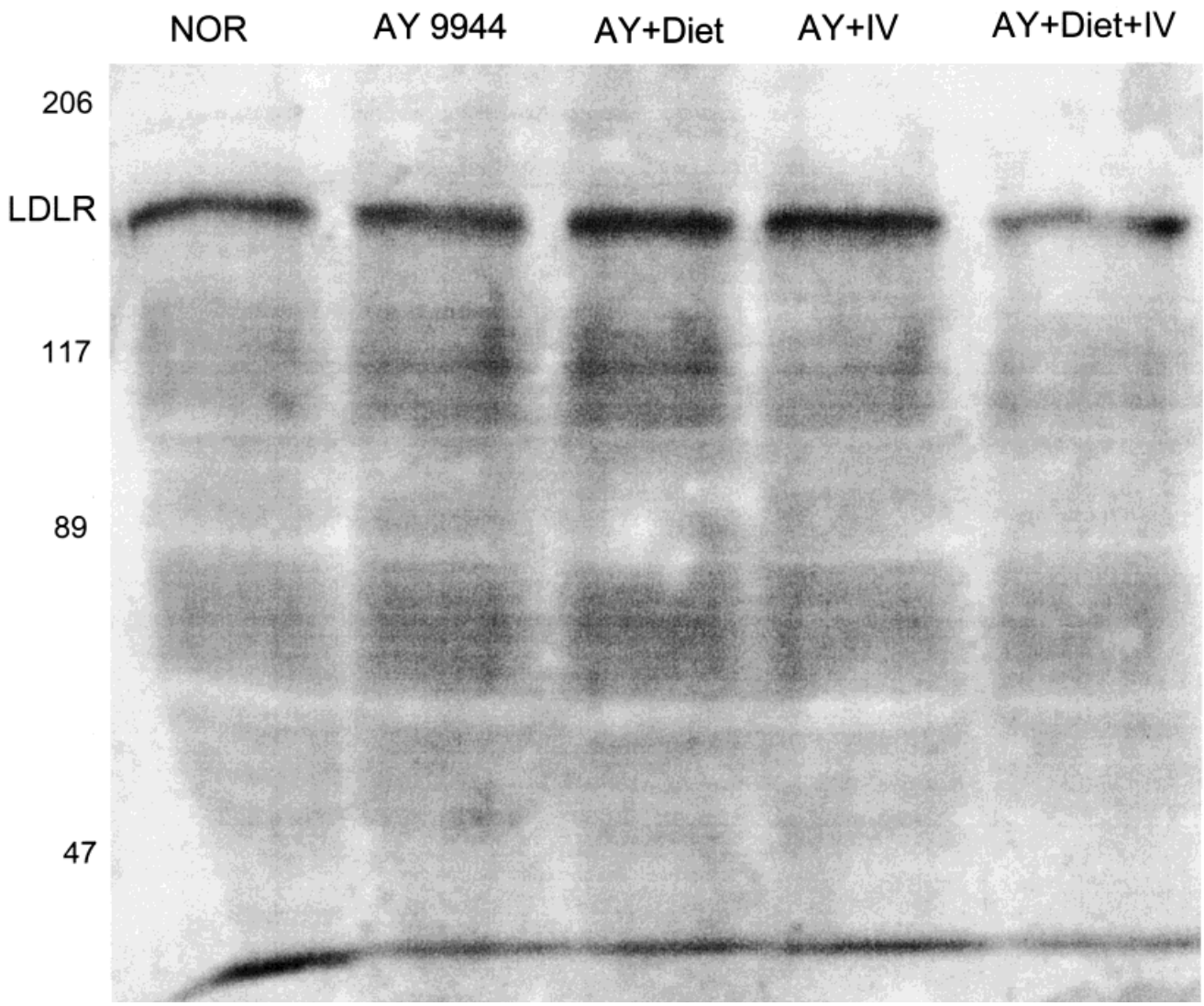

Fig. 3. Effects of dietary and intravenously administered cholesterol on hepatic LDL receptor immunoreactive protein. Experimental conditions are the same as those in Figure 2. Immunoreactive LDL receptor protein was detected as described under Methods.

gether with dietary cholesterol may be helpful in raising serum and tissue cholesterol levels in SLO patients. It has been shown that the survival of SLO patients is strongly correlated with their plasma cholesterol levels [Tint et al., 1995]. The presently used dietary cholesterol treatment generally produces only modest increases in serum cholesterol levels [Irons et al., 1995]. Although brain cholesterol levels were not significantly affected by the treatments employed in this study, the possibility that treatment of much younger AY 9944-fed rats with intravenous lipoprotein cholesterol may significantly increase brain cholesterol needs to be examined.

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REFERENCES

Irons M, Elias ER, Abuelo D, Tint GS, Salen G (1995): Clinical features of the Smith-Lemli-Opitz syndrome and treatment of the cholesterol metabolic defect. Int Pediatr 10:28–32.

Leonard DA, Kotarski MA, Tessiatore JE, Favata MF, Trzaskos JM (1994): Posttransciptional regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by 3β-hydroxy-lanost-8-en-32 al, an intermediate in the conversion of lanosterol to cholesterol. Arch Biochem Biophys 310:152–157.

Lowry RB, Young SL (1980): Borderline intelligence in the Smith-Lemli-Opitz syndrome. Am J Med Genet 5:413–425.

Natowicz MR, Evans JE (1994): Abnormal bile acids in the Smith-Lemli-Opitz syndrome. Am J Med Genet 50:364–367.

Ness GC, Pendleton LC, Pendleton AS (1987): Loss of NADPH during assays of HMG-CoA reductase: Implications and approaches to minimize errors. Lipids 22:409–412.

Ness GC, Keller RK, Pendleton LC (1991): Feedback regulation of hepatic 3-hydroxy-3-methlyglutaryl coenzyme A reductase activity by dietary cholesterol is not due to altered mRNA levels. J Biol Chem 266:14854–14857.

Ness GC, Eales S, Lopez D, Zhao Z (1994): Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression by sterols and nonsterols in rat liver. Arch Biochem Biophys 308:420–425.

Ness GC, Zhao Z, Lopez D (1996): Inhibitors of cholesterol biosynthesis increase hepatic low density lipoprotein receptor protein degradation. Arch Biochem Biophys 325:242–248.

Ness GC, Lopez D, Borrego O, Gilbert-Barness E (1997): Increased expression of low density lipoprotein receptor in a Smith-Lemli-Opitz infant with elevated bilirubin levels. Am J Med Genet 68: 294–299.

Salen G, Shefer S, Batta AK, Tint GS (1995): Biochemical abnormalities in the Smith-Lemli-Opitz syndrome. Int Pediatr 10:33–36.

Smith DW, Lemli L, Opitz JM (1964): A newly recognized syndrome of multiple congenital anomalies. J Pediatr 64:210–217.

Tint GS, Irons M, Elias ER, Batta AK, Frieden R, Chen TS, Salen G (1994): Defective cholesterol biosynthesis associated with the Smith-Lemli-Opitz syndrome. N Engl J Med 330:107–113.

Tint GS, Salen G, Batta AK, Shefer S, Irons M, Elias ER, Abuelo DN (1995): Correlation of severity and outcome with plasma sterol levels in variants of the Smith-Lemli-Opitz syndrome. J Pediatr 127:82–87.

Trzaskos JM (1995): Oxylanosterols as modifiers of cholesterol biosynthesis. Prog Lipid Res 34:99–116.

Xu G, Salen G, Shefer S, Ness GC, Chen TS, Zhao Z, Tint GS (1995): Reproducing abnormal cholesterol biosynthesis as seen in Smith-Lemli-Opitz syndrome by inhibiting the conversion of 7-dehydrocholesterol to cholesterol in rats. J Clin Invest 95:76–81.